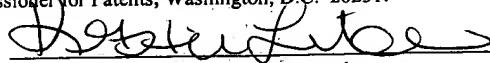
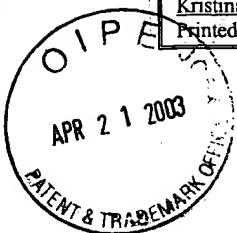


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PATENT
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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: Xinnian Dong *et al.* Art Unit: 1638
 Serial No.: 08/908,884 Examiner: A. Nelson
 Filed: August 8, 1997 Customer No.: 21559
 Title: ACQUIRED RESISTANCE GENES AND USES THEREOF

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DECLARATION OF FREDERICK M. AUSUBEL, Ph.D.

I declare:

1. I am a co-inventor of the pending claims in the above-referenced application.
2. I am a Professor of Genetics at Harvard Medical School and a Molecular Biologist in the Department of Molecular Biology at Massachusetts General Hospital. I am also a member of the National Academy of Sciences. I am an author of over 200 publications in the fields of genetics and molecular biology, and I have served on a number of editorials boards, including *Journal of Bacteriology* (1984 to 1991), *Molecular Plant-Microbe Interactions* (1987-1994; Editor in Chief 1992-1994), *Annual Review of Genetics* (1987-1991), *Current Biology* (1993 to 1995), *Genes to Cells* (1995 to 1998), *Current Protocols in Molecular Biology* (1986 to present), *Current Opinion in Plant*

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Biology (1998 to present), *Genome Biology* (1999 to present), *Plant Physiology* (2000 to present), and *Proceedings of the National Academy of Sciences, USA* (2001 to present).

3. I have read the Office Action mailed on October 18, 2002, and I have considered the Office's remarks regarding the teachings of the specification with respect to the written description and enablement of the claimed subject matter. In my opinion, these concerns are unwarranted.

4. With respect to the concern expressed by the Patent Office that focuses on description of structural features and characteristics that distinguish NPR, I note that amino acids numbered 262-289 and 323-371 of the translated NPR1 protein sequence show homology to a mouse ankyrin protein and an ankyrin-repeat motif, respectively (see, for example, Figure 5 of the present application). Figure 6A of the application shows the alignment of the NPR1 amino acid sequence with mouse ankyrin 3 (ANKB), and Figure 6B shows the alignment of the ankyrin repeats in NPR1 with the ankyrin repeat consensus derived from Michael and Bennett (*Trends in Cell Biology* 2:127-129, 1992) and Bork (*Proteins: Structure, Function, and Genetics* 17:363-374, 1993). As discussed on pages 36-41 and 47 of the specification, the *npr1-1* mutant in which the conserved histidine in the third ankyrin repeat consensus is replaced by a tyrosine displays susceptibility to virulence pathogens even after SAR induction. This

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mutant phenotype was complemented by a comsid contig containing the NPR1 gene. In describing this mutant, Famodu *et al.* (WO 00/28036, Exhibit 2) states:

[t]he lesion in one *npr1* mutant allele disrupted the ankyrin consensus sequence, suggesting that these repeats are important for NPR1 function. Furthermore, transformation of the cloned wild-type *npr1* gene into *npr1* mutants not only complemented the mutations, restoring the responsiveness to SAR induction with respect to PR-gene expression and resistance to infections, but also rendered the transgenic plants more resistant to infection by *P. syringae* in the absence of SAR induction (Cao et al. (1997) cell 88:57-63) (page 1, line 34 through page 1, line 2).

Thus, the ankyrin repeat is an important motif for structurally defining this novel class of acquired resistance genes.

Given its molecular role in disease resistance, the claimed family of disease-resistance polypeptides are readily distinguishable from unrelated ankyrin-repeat-containing polypeptides that have been described in the literature, which, to the best of my knowledge, have not been shown to possess this property. Moreover, because acquired resistance plant defense responses are ubiquitous in the plant kingdom, and because we have demonstrated that an ankyrin-repeat-containing polypeptide controls the onset of such responses in *Arabidopsis*, it is entirely reasonable to assume that other plants possess and express such genes to regulate disease resistance. Based on applicants' description, one skilled in the art would immediately recognize that this description encompasses — not one gene — but a family of genes encoding ankyrin-repeat-containing, disease resistance polypeptides.

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For example, post-filing evidence, presented by Bougri *et al.*,¹ demonstrates that acquired resistance genes from wheat, corn, and rice share “significant sequence homology in the region of ankyrin repeats” with the *Arabidopsis* NPR1 acquired resistance gene, signifying that this structural feature is common to members of the genus. Thus, one skilled in the art would appreciate that other nucleic acids that hybridize to the *Arabidopsis* NPR1 gene also encode ankyrin-repeat-containing, disease resistance polypeptides.

5. I have also considered the Office’s concern that the identification of additional NPR family members would require undue experimentation. Given the teaching of the specification and the level of skill known in the art at the time the present application was filed, genes falling within the scope of the present claims could routinely be identified and isolated from a variety of plant sources using nothing more than standard techniques of molecular biology.

With respect to gene isolation methodologies, clear instructions for isolating other claimed nucleic acid molecules are provided in the specification under the heading “Isolation of Other Acquired Resistance Genes,” at pages 50-52. There, the specification sets forth general guidance on the routine methods known at the time the application was filed for identifying the gene sequences required by the claims. These standard cloning

¹ See Bougri *et al.*, Acquired Resistance Genes in Plants, WO 00/70069 (Exhibit 1), page 32 (page 32, lines 15-17).

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methods described in the specification include: (1) the design and utilization of oligonucleotides for cloning acquired resistance gene sequences, (2) low- and high-stringency hybridization cloning methodologies, (3) library screening procedures, and (4) PCR-based amplification cloning strategies. Using such techniques, genes falling within the claims may be readily isolated, absent undue experimentation, from virtually any plant using applicants' *NPR1* sequence as a starting material.

Any "experimentation" involved in isolating and characterizing additional nucleic acid molecules falling within the present claims is straightforward, and is rendered so by our discovery of the sequence encoding *NPR1*. Specifically, if one skilled in the art wished to isolate homologous NPB sequences from other plants, they would simply use the disclosed *NPR1* nucleotide sequences as a probe in combination with conventional gene screening methods, such as hybridization. These approaches would require only standard applications of hybridization wash conditions, and possibly the type of empirical condition adjustments carried out routinely, and successfully, by molecular biologists in isolating a gene. In addition, I note that methods for screening recombinant libraries, as well as methods for determining the sequence of an isolated clone, had been routinely used in the art for over 20 years prior to the filing of our application. For example, in 1975 and 1977, Grunstein and Hogness² and Benton and Davis,³ respectively, provided

² Grunstein and Hogness, Colony hybridization: A method for isolating of cloned DNAs that contain a specific gene. *Proc. Natl. Acad. Sci. U.S.A.* 72: 3961, 1975 (Exhibit 4).

³ Benton and Davis, Screening λ gt recombinant clones by hybridization to single plaques *in situ*. *Science* 196: 180, 1977 (Exhibit 5).

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methods for isolating specific genes from recombinant libraries, and, by at least 1977, Sanger, Nicklen, and Coulson⁴ enabled methods for determining the DNA sequences of such isolated genes.

The nature of molecular biology is that it involves screening recombinant libraries to determine which clone within a library contains the gene with the desired characteristics. Practitioners in the art of molecular biology are prepared to screen many clones to find one that contains a desired gene. Screening of a recombinant library to isolate an NPR gene sequence is considered to be a routine step in the process of isolating a gene having desired characteristics; it does not constitute undue experimentation.

In addition, once isolated, these gene sequences may be subjected to standard DNA sequencing to confirm their structural relatedness to the disclosed *NPR1* gene and its encoded ankyrin-repeat-containing polypeptide. If desired, publicly available sequence analysis software may be utilized for rapidly identifying the ankyrin-repeats. All of the above methods are routinely used in the art of molecular biology and that all were well established at the time our application was filed.

In addition, as further evidence that genes encoding ankyrin repeat-containing, disease resistance polypeptides may be isolated using nothing more than standard techniques, the Examiner is directed to the present specification, for example, at pages 49-50. There, under the heading "Isolation of Solanaceous AR Genes," the specification

⁴ Sanger, Nicklen, and Coulson, DNA sequencing with chain-terminating inhibitor. *Proc. Natl. Acad. Sci. U.S.A.* 74: 5464, 1977 (Exhibit 6).

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describes the successful and straightforward isolation of an *NPR1* homolog from tobacco. This homolog was identified by screening a cDNA library with a probe prepared from the full-length *Arabidopsis NPR1* cDNA. The isolated solanaceous acquired resistance gene, like the cruciferous *NPR1* gene, was found to encode an ankyrin-containing polypeptide. In addition, the tobacco *NPR1* homolog shows significant sequence identity to the *Arabidopsis* *NPR1* gene product. Consistent with these results in tobacco are the results described in the present specification at page 52 (lines 4-15). There, results of an RNA blot experiment are described that demonstrate the existence of yet another *NPR1*-hybridizing RNA, in this case, in potato.

Such data strongly corroborate the assertion that structurally related gene sequences exist and that they may be identified and isolated from a variety of plant sources using applicants' *NPR1* sequence and standard techniques that are both described in the present specification and known in the art. In particular, the guidelines provided by the teachings of our specification have been effective for such gene identification from at least two plants other than *Arabidopsis*, and a plant family other than crucifers, and there is no reason to believe that *NPR1* homologs cannot similarly be identified from any number of other sources.

With respect to the further issue of whether such genes would confer disease resistance, I again refer to the present specification. As taught, for example, at page 69 (lines 15-17), the ability of a structurally related gene to confer plant disease resistance is easily established using any of a variety of methods, including a straightforward, one-step

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screening technique. The specification makes clear that broad-spectrum pathogen resistance is readily obtained by expressing an acquired resistance transgene to initiate a plant defense response. Moreover, at pages 45-46, the specification demonstrates that overexpression of a *35S-NPRI* transgene in *Arabidopsis* conferred resistance on the plant to bacterial and fungal pathogens. Accordingly, a skilled worker need only prepare transgenic plants overexpressing a gene found to be structurally related to *NPRI*, and then evaluate the plant's ability to combat a pathogen. Such a single-step screening approach does not constitute undue trial and error experimentation.

Additionally, I note that the Examiner grounds the rejection on the assertion that there is no "definitive evidence demonstrating the existence of a structurally related DNA encoding a polypeptide comprising an ankyrin repeat motif." This assertion is incorrect. On this point, I again direct the Examiner's attention to Bougri's ankyrin repeat containing Npr homologs. In particular, at page 52, where under the heading "Analysis of transgenic rice for enhanced resistance," Bougri states that "[t]ransgenic overexpression of *Nph1* and *Nph2-1*⁵ Npr homologs promotes strong resistance against *M. grisea*." Here Bougri, after identifying the ankyrin repeat containing rice and wheat Npr homologs, respectively *Nph1* and *Nph2-1*, overexpressed the wheat and rice Npr homologs, independently, in rice. Overexpression of these Npr homologs resulted in lines of rice having resistance to fungal blast disease (see Bourgri, at page 52, lines 25-

⁵ The *Nph1* and *Nph2-1* genes are described by Bougri (WO 00/70069; Exhibit 1) at page 8 (lines 15-22) as being *Npr1* homologs respectively from rice and wheat.

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26).⁶ Further confirming applicants' teaching, Bougri, at page 52 (lines 26-27), states that "these results suggest that both wheat and rice Npr homologs, when expressed in rice, enhance the SAR pathway." Thus, Bourgri not only corroborates that our claimed acquired resistance gene family encodes polypeptides having an ankyrin repeat, but also corroborates that such proteins, when overexpressed in rice, confer enhanced disease resistance to a plant pathogen.

Furthermore, Crane *et al.* (U.S.P.N. 6,504,084, Exhibit 3) describes the isolation of the NPR gene from maize. One of the maize sequences was identified as part of the NPR gene based on its homology to the NPR1 gene. In particular,

Crane states:

[a] BLAST search of the Pioneer Hi-Bred Int'l Inc. propriety database identified a sequence, 798034, as homologous to NPR1. CJRMC70, the longest fragment was used to screen a lambda cDNA library made from 6 inch maize seedlings (genotype B73) cloned into _ Express (Stratagene; La Jolla, Calif.). (column 45, lines 48-53).

In characterizing this gene, Crane reports:

[p]rotein sequence homology was calculated based on PILEUP alignment of derived amino acid sequences of *Arabidopsis* NPR1 and maize NPR1. DNA coding-region homology was estimated from an alignment of the open reading frames based on the amino acid PILEUP alignment. The cDNA alignment showed 1,677 positions aligning (not counting the gaps) with 824 positions containing the same base (49.1% identity). The alignment of the amino acid sequences derived from the cDNA sequence of *Arabidopsis* NPR1 and maize NPR1 shows a total of 579 positions aligning (not counting gaps) and 222 positions (38.3% identity) with identical amino acids and 302 positions (52.2% similarity) with identical or similar amino acids. (column 46, lines 23-36).

⁶ I note that Bougri (WO 00/70069; Exhibit 1), at page 50 (lines 27-28), notes that one of the wheat Npr homologs, *Nph2-1*, "[did] not appear to promote disease resistance in [transgenic] wheat."

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Famodu reports the identification of several NPR1 genes, from corn, rice, and wheat, using the *Arabidopsis thaliana* NPR1 gene. In particular, Famodu states that "cDNA clones encoding NPR1s were identified by conducting BLAST ... searches for similarity to sequences contained in the BLAST 'nr' database" (page 16, lines 6-9). Under the header "Characterization of cDNA Clones Encoding NPR1" Famodu further states that "[t]he BLASTX search using EST sequences from clones listed in Table 3 revealed similarity of the polypeptides encoded by the cDNAs to NPR1 from *Arabidopsis thaliana* (NCBI General Identifier No. 1773295)" (page 16, lines 25-27). Based on this analysis, Famodu concludes:

[s]equence alignments and BLAST scores and probabilities indicate that nucleic acid fragments comprising the instant cDNA clones encode a substantial portion of one corn, two rice, and one wheat NPR1. These sequences represent the first corn, rice, and wheat sequences encoding NPR1. (page 17, lines 16-19).

Given this evidence of rice, wheat, maize, and corn Npr homologs, there is no scientific reason for doubting the existence of the claimed gene family of disease resistance polypeptides. This evidence also demonstrates the ability of skilled artisans to readily identify additional NPR genes based on the structural characteristics of their sequence homology to the *Arabidopsis* NPR1 gene provided by applicants' specification and the presence of an ankyrin repeat motif.

At the time of filing, a skilled artisan, using no more than routine experimentation and the teachings of the present specification, could easily screen structurally related

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genes to determine the level of resistance provided by any particular ankyrin-repeat-containing polypeptide against a plant pathogen. Such screening could easily be accomplished using standard techniques for generating plants expressing such proteins and thus does not constitute undue experimentation.

I note that for all of the aforementioned reasons no scientific evidence currently made of record in this case establishes a basis for doubting the objective truth of the statements found in the specification regarding enablement with respect to isolating genes falling within the present claims and determining whether such genes possess disease resistance properties. As is discussed above, our statement that expression of an acquired resistance gene encoding a polypeptide possessing an ankyrin repeat confers pathogen resistance on host plants is in accordance with the evidence described in the present specification for the *NPR1* gene and the post-filing evidence of Bougri. Moreover, given the evidence described above, the Examiner has provided no evidence or reason for doubting our statement that other genes having the structural features described by applicants would function similarly as disease resistance genes.

6. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001

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of Title 18 of the United States Code and that such willful false statements may
jeopardize the validity of the application of any patents issued thereon.

Date: April 16, 2003


Frederick M. Ausubel
Frederick M. Ausubel, Ph.D.